Clustered array of ochratoxin A biosynthetic genes in *Aspergillus steynii* and their expression patterns in permissive conditions

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Abstract

Aspergillus steynii is probably the most relevant species of Section Circumdati producing ochratoxin A (OTA). This mycotoxin contaminates a wide number of commodities and it is highly toxic for humans and animals. Little is known on the biosynthetic genes and their regulation in *Aspergillus* species. In this work, we identified and analyzed three contiguous genes in *A. steynii* using 5'-RACE and genome walking approaches which predicted a cytochrome P450 monoxygenase (*p450ste*), a non-ribosomal peptide synthetase (*nrpsste*) and a polyketide synthase (*pksste*). These three genes were contiguous within a 20742 bp long genomic DNA fragment. Their corresponding cDNA were sequenced and their expression was analyzed in three *A. steynii* strains using real time RT-PCR specific assays in permissive conditions in *in vitro* cultures. OTA was also analyzed in these cultures. Comparative analyses of predicted genomic, cDNA and amino acid sequences were performed with sequences of similar gene functions. All the results obtained in these analyses were consistent and point out the involvement of these three genes in OTA biosynthesis by *A. steynii* and showed a co-ordinated expression pattern. This is the first time that a clustered organization OTA biosynthetic genes has been reported in *Aspergillus* genus. The results also suggested that this situation might be common in *Aspergillus* OTA-producing species and distinct to the one described for *Penicillium* species.

Keywords

Polyketide synthase, non-ribosomal peptide synthetase, cytochrome p450 monooxygenase, *Aspergillus steynii*, ochratoxin A, biosynthetic cluster

1. INTRODUCTION

Ochratoxin A (OTA) is a secondary metabolite produced by several *Aspergillus* and *Penicillium* species (El Khoury and Atoui, 2010). Among other toxic effects, it has potent nephrotoxic properties and has been classified as a possible human carcinogen (group 2B) by the International Agency for Research in Cancer (IARC) (IARC, 1993). OTA is a widespread mycotoxin and it is frequently found in many common substrates in human diet such as cereals, grapes and derivatives, coffee, fruits, nuts and spices as well as in other non-conventional sources (Covarelli et al. 2012; Duarte et al. 2009, 2010b; El Khoury and Atoui, 2010). According to the risk this mycotoxin might pose for human health, the maximum OTA limits allowed in different agrofood products are regulated in many countries, including the European Union (Duarte et al. 2010a; European Commission, 2006, 2010).

Aspergillus ochraceus was considered for a long time the main OTA producer of *Aspergillus* section Circumdati (Pitt, 2000). However, Frisvad et al. (2004) reported *A. steynii* and *A. westerdijkiae* as two new species included in this section which were able to produce OTA. In our group, the ability of these three species to produce OTA has been confirmed and, particularly *A. steynii* seemed to be the most important OTA producer of section Circumdati. Most of the strains of this species were capable of producing the toxin at levels 100 and 1000 times higher than those reached by *A. westerdijkiae* and *A. ochraceus*, respectively (Gil-Serna et al. 2011). Due to their recent description, the presence of *A. steynii* in food matrices has not been extensively studied although it has been reported as an important OTA-producing contaminant of coffee (Leong et al. 2007; Noonim et al. 2008) and barley (Mateo et al. 2011).

The molecule of OTA is formed by a dihydroisocoumarin moiety linked to a phenylalanine by an amide linkage (IUPAC, 1992). Despite the importance of this toxic

secondary metabolite, the enzymes involved in the different steps of its biosynthetic pathway as well as its genetic control are far from being fully known in any fungal species. Different hypotheses regarding OTA biosynthesis have been published although the most accepted is that proposed by Huff and Hamilton (1979). The isocoumarin group would be formed via a polyketide synthesis pathway starting from acetate and malonate and the phenylalanine would be derived from the shikimic acid pathway. According to this proposed pathway, several enzymes might be involved such as a polyketide synthase (PKS), a non-ribosomal peptide synthetase (NRPS), a cytochrome p450 monooxygenase (P450), a chloroperoxidase (CHL) and an esterase. There is little knowledge on the encoding genes for the putative OTA biosynthetic enzymes and only few full DNA sequences have been reported so far. The most advanced research regarding OTA biosynthesis has been reported in *Penicillium* genus. Three genes have been identified encoding a PKS (*otapksPN*, 2.2 kb, accession number AY557343.2), a NRPS (*npsPN*, 2.2 kb, AY557343.2) and a CHL (*otachlPN*, 951 bp) in *P. nordicum* (Geisen et al. 2006; Karolewiez and Geisen, 2005) and a PKS (*otapksPV*, 1 kb, DQ789993.1) in *P. verrucosum* (Abbas et al. 2013; Schmidt-Heydt et al. 2007). Gene disruption experiments confirmed that *otapksPN* and *otapksPV* were involved in OTA biosynthesis in *P. nordicum* and *P. verrucosum*, respectively (Karolewiez and Geisen, 2005; Abbas et al. 2013). In the case of OTA-producing species from *Aspergillus* genus only few sequences have been characterized as related to OTA biosynthesis. The first gene identified corresponded to a partial sequence of a PKS encoding gene (*pks*, 1.5 kb, AY272043) in *A. ochraceus* (currently *A. westerdijkiae*) and its involvement in OTA biosynthesis was confirmed by gene inactivation (O'Callaghan et al. 2003). Subsequently, two partial sequences were identified which encoded a PKS (*aoks1*, 2 kb, AY583209.1) and a P450 (*p450-B03*, 463 bp, DQ054596) in *A.*

westerdijkiae (Bacha et al. 2009; O'Callaghan et al. 2006) as well as a PKS (*ACpks*, 2.2 kb, AM944567.1) and a NRPS (*AcOTAnrps*, 5.7 kb) in *A. carbonarius* (Gallo et al. 2009, 2012). The disruption of *AcOTAnrps* and *aoks1* genes in *A. carbonarius* and *A. westerdijkiae*, respectively, produced mutant isolates which were not able to produce OTA (Bacha et al. 2009; Gallo et al. 2012).

Many of the fungal genes which encoded enzymes involved in biosynthetic pathways of secondary metabolites, including mycotoxins, are clustered in the same genome location such as aflatoxins (Yu et al. 2004), trichothecene or fumonisin biosynthetic genes (Desjardins and Proctor, 2007). In *P. nordicum* two genes involved in OTA biosynthesis, *otapksPN* and *otanpsPN*, have been reported so far to be located consecutively in the fungal genome (Karolewiez and Geisen, 2005). Recently, Gallo et al. (2014) also reported an OTA *pks* encoding gene located downstream of the *AcOTAnrps*. The chromosomal location of the OTA biosynthetic genes described in any of the *Aspergillus* section Circumdati species above mentioned is not known yet.

On the basis of the sequence of the *p450-B03* gene putatively involved in OTA biosynthesis by *A. westerdijkiae* as a starting point, the objectives of this work were (i) to identify and characterize genes involved in OTA biosynthesis in *A. steynii*, (ii) to determine their expression pattern in relation with OTA biosynthesis and (iii) to establish their putative location in a cluster.

2. MATERIALS AND METHODS

2.1. Fungal strains and culture conditions

Three *A. steynii* strains were used in this work. *A. steynii* 3.53 and Aso2 were isolated from coffee and grapes and they were kindly supplied by Dr Sanchis (University of Lleida, Spain) and Dr Jimenez (University of Valencia, Spain), respectively. Both

strains showed high levels of OTA production (Gil-Serna et al. 2011). Additionally, *A. steynii* CBS 112813, obtained from the Centralbureau voor Schimmel culture collection (The Netherlands), was used as negative control since it was reported as a non-producing isolate (Gil-Serna et al. 2011). The correct identification of the three strains was confirmed and reported previously (Gil-Serna et al. 2009).

The strains were maintained as spore suspension in 15% glycerol at –80ºC. Spore suspensions were prepared from a 5-day-old sporulating culture of the corresponding *A. steynii* strains in Czapek Dox Modified Agar (Pronadisa, Spain). Spores were harvested and resuspended in saline solution (9 g/L sodium chloride) and concentration was measured using a Thoma counting chamber. The spore suspensions were diluted when necessary.

2.2. Nucleic acid extraction

2.2.1. DNA extraction

A. steynii strain 3.53 was cultured in 100 ml Erlenmeyer flasks with 20 ml of Sabouraud Broth (Pronadisa, Spain) at 28±1ºC on orbital shaker (120 rpm) for 2 days. Mycelia was harvested by filtration through Whatman paper nº1 and kept at -80ºC for DNA isolation. Filtrate mycelia were frozen with liquid nitrogen and grinded using a mortar and a pestle. Genomic DNA extraction was performed starting from 100 mg of grinded mycelia following the protocol described by Querol et al. (1992).

2.2.2. RNA extraction and cDNA synthesis

RNA was obtained from cultures of *A. steynii* strains 3.53, Aso2 and CBS 112813 in plates with CYA medium (45.4 g/l Czapek-Dox Modified Agar, 5 g/l yeast extract). To enable the easier removal of the mycelia, sterile cellophane membranes (P400, Cannings Ltd, United Kingdom) were laid on the plates before fungal inoculation. Two

151 µl of a spore suspension (10^7 spores/ml) were located in the centre of CYA plates and
152 the incubation was performed at 28 ºC. Mycelia were harvested between 3 and 8 days of
153 incubation and frozen at -80 ºC before RNA extraction. RNeasy Plant Mini Kit
154 (QIAgen, Spain) was used following manufacturer's instruction starting from 100 mg of
155 frozen mycelia grinded using a mortar and a pestle. Subsequently, DNA was removed
156 from isolated RNA by two treatments with DNase I (QIAgen, Spain). cDNA was
157 prepared starting from 1 µg of isolated RNA using a reverse-transcriptase enzyme
158 (Roche, Spain) primed using random hexamers (Promega, Spain). Two biological
159 replicates were performed for each strain and day of incubation.
160 Nucleic acid (DNA and RNA) concentrations were determined using a NanoDrop®
161 ND-1000 spectrophotometer (Nanodrop Technologies, USA).
162

163 **2.3. PCR amplification, cloning and sequencing**

164 All PCR amplifications performed in this work were carried out in an Eppendorf
165 Mastercycler Gradient (Eppendorf, Germany) in volumes of 25 µl containing 17.15 µl
166 of molecular biology water (MO-BIO, USA), 2.5 µL of 10x PCR buffer, 1 µL of $MgCl_2$
167 (50 mM), 0.2 µL of dNTPs mix (100 mM) (Ecogen, Spain), 1 µL of each primer (20
168 µM) (IDT, Spain) and 0.15 µL of Taq DNA polymerase (5 U/µL) (Biotools, Spain).
169 Two µl of DNA at a concentration of 50 ng/µl were added to the master mix in each
170 case.
171 PCR products were detected in agarose ethidium bromide gels in TAE 1X buffer (Tris-
172 acetate 40 mM and EDTA 1.0 mM) at the appropriate percentage of agarose (1-2.5%).
173 The GeneRuler 1 kb DNA ladder (Biotools, Spain) was used as molecular size marker.
174 Bands were purified from the gel before cloning using the QIAquick Gel Extraction Kit
175 (QIAgen, Spain).

176 The pGEM®-T Vector System I (Promega, USA) was used to clone PCR products
177 before sequencing following the supplier's protocol. Transformation was performed
178 using High Efficiency *E. coli* JM109 Competent Cells (Promega, USA). Plasmid DNA
179 purification from transformed colonies was carried out with the NucleoSpin® Plasmid
180 Kit (Macherey-Nagel, Germany) according to manufacturer's instructions.
181 Both strands of cloned PCR products were sequenced by STAB Vida Lda. (Portugal)
182 using an ABI 3730XL sequencer (Applied Biosystems, USA). Sequences were edited
183 with EditSeq and MegAlign programs included in DNAstar 7.1 software (Lasergene,
184 USA). The edited sequences were compared with those previously available on the
185 NCBI databases using the BLAST program.
186
187 **2.4. Amplification of a gene encoding a P450 in *Aspergillus steynii***
188 The complete ORF encoding a putative P450, *p450-B03* gene, involved in OTA
189 biosynthesis in *A. westerdijkiae* had been obtained previously in our group (González-
190 Salgado, 2009). The corresponding genomic DNA and cDNA sequences were 1771 and
191 1545 bp long, respectively. On the basis of these sequences, several primer pairs were
192 designed to amplify the 3' end of a similar gene in *A. steynii*. The primer set
193 OCRAp450F (5'-TTTGGCATTGCGGAACTTTAC-3') and OCRAp450R (5'-
194 CATGTCAAACGTGCGGAAGA-3') was able to produce a 58 bp fragment using
195 either *A. westerdijkiae* or *A. steynii* genomic DNA. The PCR product obtained from *A.*
196 *steynii* was cloned and sequenced as described above. The sequence obtained showed a
197 93% of identity with the corresponding sequence of *p450-B03* gene of *A. westerdijkiae*.
198 The upstream sequence of the 58 bp fragment was obtained in *A. steynii* using the 5'-
199 RACE approach with the kit 5´ RACE System for Rapid Amplification of cDNA Ends
200 (Invitrogen, Canada) following the manufacturer's instructions. The first PCR was

performed using the primers GSP1 and GSP1p450ste (5'-GTCAAACGTGCGGAAGA-3'), whereas nested PCR was carried out with GSP2 and GSP2p450ste (5'-CGTGGCAACAGCCATGTAAAGT-3') and the conditions specified in the protocol. GSP1 and GSP2 primers were included in the selected kit. The 1.4 kb product obtained after nested PCR was cloned and sequenced as described above. Subsequently, the complete sequence of the DNA of the gene encoding a P450 in *A. steynii* 3.53 was obtained as well using new primers developed on the basis of the cDNA sequence obtained by 5'-RACE. The amplification was performed using p450steCOMP-F (5´-GAACAGCAAGAAGCATGGACTT-3') and p450steCOMP-R (5´-CATGTCAAACGTGCGGAAGACC-3´) and the conditions were as follows: 5 min at 95 ºC, 40 cycles at 95 ºC for 45 s, 50 ºC for 45 s and 72 ºC for 1 min 30 s and finally a final extension at 72 ºC for 7 min. The PCR product was purified, cloned and sequenced.

2.5. Genome Walking

A Genome Walking Approach was followed to study the flanking regions of the *p450ste* gene using the GenomeWalker™ Universal Kit (Clontech Laboratories Inc., USA). Libraries were constructed with four restriction enzymes (Dra I, EcoR V, Pvu II and Stu I) and amplifications were performed with the primary and nested primers supplied by the manufacturer (AP1 and AP2, respectively) and those designed on the basis of the sequence obtained in each round. All primers used in each round of Genome Walking are indicated in table 1. In each round, the longest PCR fragment obtained after nested amplification from at least one of the four libraries was gel purified, cloned and sequenced as described before.

2.6. Quantification of gene expression by real-time RT-PCR

The expression of the three genes identified by genome walking in *A. steynii* as well as of a internal control gene, encoding ß-tubulin were studied using new specific real time RT-PCR protocols. RNA extraction and cDNA preparation were performed as described above. Gene expression of the three genes was quantified in two OTA-producing strains (3.53 and Aso2) and one non-producing isolate (CBS 112813). Normalized quantification was performed and all data were shown related to the expression of the constitutive ß-tubulin gene (*ßtub*) used as endogenous control. The calibrator sample corresponded to the value of expression of the non-producing strain after 3 days of incubation. In all cases, gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). This method can only be used if the difference in amplification efficiencies between the constitutive and target gene is less than 10% (Schmittgen and Livak, 2008).

The primer sets were designed on the basis of the cDNA sequences of this work or the *ßtub* sequences obtained in previous studies performed in our group (Gil-Serna et al. 2009). The sequences of these primers used for real time PCR and their amplification efficiencies are shown in table 2. The optimization of each protocol was tested by generating a standard curve starting from ten-fold serial dilution of DNA from *A. steynii* 3.53 and Aso2 strains (from 50 to $5x10^{-3}$ ng/µl). Three parameters (amplification efficiency, correlation coefficient and dissociation curve) were analyzed to check the correct optimization of the method.

The assays were carried out and monitored in an ABI PRISM 7900HT system (Applied Biosystems, Spain) in the Genomic Unit of the Complutense University of Madrid. The final reaction volume (10 µl) contained: 5 µl FastStart Universal SYBR Green Master (Rox)(Roche, Spain), 0.6 µl forward primer 5 µM (IDT, Spain), 0.6 µl reverse primer 5

µM (IDT, Spain), 2.5 µl cDNA template prepared as described above and 1.3 µl molecular grade water. The real time RT-PCR assays were performed using a standard program: 95 ºC for 10 min, 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. All reactions were carried out by duplicate.

2.7. OTA analysis

OTA production of *A. steynii* strains was evaluated in the same CYA plates used for RNA extraction after removing the cellophane with the fungal mycelia. OTA extraction was performed by a method described elsewhere (Bragulat et al. 2001) and OTA concentration was measured by High Performance Liquid Chromatography (HPLC) on a reverse phase C18 column (Tracer Extrasil ODS2; 5 µm, 4.6 mm x 250 mm; Teknokroma, Spain) at 45 ºC in a Perkin Elmer Series 200 HPLC system coupled with a fluorescence detector (Perkin Elmer, USA) at excitation and emission wavelengths of 330 and 470 nm respectively. The mobile phase contained monopotassium phosphate 4 mM pH 2.5 and methanol (33:67) and the flow rate was 1 ml/min. OTA was eluted and quantified by comparison with a calibration curve generated from OTA standards (OEKANAL®, Sigma-Aldrich, Steinheim, Germany).

2.8. Statistical analysis

Statistical treatment of the data obtained was performed using SPSS 19 software (IBM, USA). After checking normality and homoscedasticity of the data, a two way ANOVA was carried out to compare the expression ratio of the genes by the OTA producing and non-producing *A. steynii* strains along the time. Correlation among the expression of the three genes described in OTA producing strains along time was also checked by analysing Pearson correlation index. In all cases, statistical significance was established at $p<0.05$.

3. RESULTS AND DISCUSSION

The knowledge of the genes involved in mycotoxin biosynthetic pathways is essential since transcriptional regulation seems to be the crucial step on mycotoxin production by fungi (Miller and Linz, 2005).

In this work, we identified and described for the first time the complete sequence of three genes, and their flanking regions, putatively involved in OTA biosynthesis by *A. steynii* encoding a cytochrome P450 monooxygenase (*p450ste*), a non-ribosomal peptide synthase (*nrpsste*) and a polyketide synthase (*pksste*). These three genes were contiguous within a 20742 bp long genomic DNA fragment. The schematic representation of the relative position of *p450ste*, *nrpsste* and *pksste* in the genomic region described in *A. steynii* is shown in figure 1. The complete sequence was deposited in the NCBI nucleotide database (accession number KJ395384).

The ORF of the *p450ste* gene and its cDNA were 1822 and 1542 bp long, respectively. This cDNA sequence showed an identity of 78.7% with the 1545 bp long cDNA of the *p450-B03* gene of *A. westerdijkiae* (Gonzalez-Salgado, 2009) and 59.4% with the predicted cDNA of an ORF encoding a putative P450 located in the An15c0250 contig of the complete genome of *A. niger* CBS 513.88 (locus tag An15g07900) (Pel et al. 2007). The comparison of both the ORF and cDNA sequences of *p450ste* revealed 5 introns with lengths between 47-73 bp. This complete ORF sequence showed 72.1% similarity with that reported for *A. westerdijkiae* (*p450-B03*) (Gonzalez-Salgado, 2009) and an identity of 53.0% with the 1809 bp long ORF of the predicted P450 protein of the genome of *A. niger* mentioned above (Pel et al. 2007).

The predicted P450STE protein had 513 aminoacids and showed the characteristic motifs of this cytochrome P450 superfamily, including the three essential P450 consensus sequences: the heme-binding loop (F-X-X-G-X-R-X-C-X-G), the conserved

motif of K helix (E-X-X-R) and the proton transfer group (A/G-G-X-E/D-T-T/S) (Werck-Reichhart and Feyereisen, 2000). The identity of the amino acid sequences of the predicted P450STE protein with those predicted for P450-B03 of *A. westerdijkiae* and P450 of *A. niger* were 87.3% and 67.2%, respectively. These values decreased to less than 40% when compared to other P450 involved in the synthesis of other mycotoxins such as CYPA involved in aflatoxin synthesis pathway by *A. parasiticus* or TRI4 related to trichothecene biosynthesis in *Fusarium* species (Desjardins and Proctor, 2007; Yu et al. 2004). It seems to be widely accepted that secondary structure of P450 enzymes is conserved although the identity values among them appear to be extremely low (Cresnar and Petric, 2011; Werck-Reichhardt and Feyereisen, 2000). Several authors have studied this cytochrome P450 superfamily and proposed that all P450 proteins involved in mycotoxin biosynthetic pathways could be included in the same group (class II) (Cresnar and Petric, 2011). This class II P450 proteins are anchored on the outer face of the endoplasmic reticulum and they all contain a conserved motif with a group of prolines preceded by a cluster of basic residues (Werck-Reichhart and Feyereisen, 2000). This conserved region was also present in the predicted amino acid sequence of P450STE described in this work as well as in the predicted P450-B03 of *A. westerdijkiae*, the P450 of *A. niger* and CYPA of *A. parasiticus*; therefore, they could probably be also included in this class II of cytochrome P450 monooxygenases.

The 2.5 kb long 5' upstream region of *p450ste* ORF did not seem to contain similarity with any available sequence on databases. However, an ORF 5166 bp long was identified 1357 bp downstream the *p450ste* gene which predicted a non-ribosomal peptide synthase. This gene, named *nrpsste*, showed the highest identity (57.7%) with an ORF in the An15c0250 contig of the complete genome of *A. niger* whose sequence predicted a NRPS protein (locus tag An15g07910) (Pel et al. 2007). The cDNA

sequence obtained from the corresponding RNA isolated from *A. steynii* cultured at permissive conditions for OTA biosynthesis indicated that this *nrpsste* gene had no introns. The predicted NRPSSTE protein had 1721 amino acids and showed 54.6% of identity with the NRPS predicted by the ORF in *A. niger* genome mentioned. In both cases, the amino acid sequence showed the typical modular structures of the NRPS enzymes with the essential domains necessary to be functional (Challis and Naismith, 2004). The first module showed an adenilation (A), a peptidyl carrier protein (PCP) and a condensation (C) domains, whereas the second module only had A and PCP domains. The A domains have the 10 conserved motives which are located around the active sites of the protein as well as the conserved motives of the C domain although in some cases presented minor changes in their predicted amino acidic sequence (Eisfield, 2009). These two NRPSs lacked the thioesterase (TE) domain, considered responsible for the release of the product and frequently present in fungal NRPS (Evans et al. 2011). In contrast, the NPSPN sequence predicted from *npsPN* gene in *P. nordicum* described by Karolewiez and Geisen (2005) does not agree to a typical NRPS and is extremely different from NRPSSTE and NRPS of *A. niger*.

A third ORF was identified 873 bp downstream *nrpsste* and was 8095 bp long and predicted a polyketide synthase (PKS), which was named *pksste*. Similarly to the previously described two genes, the corresponding cDNA was obtained from RNA isolated from *A. steynii* cultures. This permitted the identification of 8 introns. As mentioned before, genes encoding PKSs involved in OTA biosynthesis have been identified in different OTA-producing species (Bacha et al. 2009; Karolewiez & Geisen, 2005; O'Callaghan et al. 2003; Schmidt-Heydt et al. 2007). The *pksste* ORF showed the highest similarity with the 1.5 kb long sequence of the first *pks* partial genomic sequence described as involved in OTA biosynthesis by *A. westerdijkiae* (O'Callaghan

et al. 2003) and the 3.4 kb long partial genomic sequence of *lc35-12* by *A. ochraceus* (Dao et al. 2005) (75.6 % and 76.6%, respectively). However, the *lc35-12* and *pks* gene sequences reported only represented the 38% and the 18% of the ORF sequence of *pksste*. Therefore, this gene described in *A. steynii* might be considered the first complete genomic sequence encoding a PKS involved in OTA biosynthesis. The best match (56% of identity) using this full genomic sequence was an 8.17 kb long ORF predicting a hypothetical *pks* gene in the An15c0250 contig (locus tag An15g07920) showing a 93% of coverage. In contrast, less than 10% identity was found when compared to the OTA biosynthetic genes reported in *Penicillium* species.

The *pksste* cDNA predicted a protein of 2553 amino acids. The highest identity (77%) was obtained with the hypothetical protein deduced from the *lc35-12* partial gene of *A. ochraceus,* which represented a coverage of 40%. The second best match was 64% of identity with the hypothetical protein ANI_1_1836134 predicted from the corresponding ORF in the genome of *A. niger* above mentioned (An15g07920) with a 99% coverage.

The analysis of PKSSTE protein sequence predicted a typical fungal multi-domain type I PKS (Chiang et al. 2010). This protein contained the essential domains of these PKSs: ketosynthase (KS), acyl transferase (AT) and acyl carries protein (ACP) (Keller et al. 2005). Other non-essential domains were also present such as dehydratase (DH), C-methyltransferase (CMeT), enoylreductase (ER) and ketoreductase (KR) being the predicted architecture of PKSSTE as follows: KS-AT-DH-CMeT-ER-KR-ACP. The number and organization of domains of the protein was the same as predicted by *A. niger* putative pks sequence (ANI_1_1836134). The presence of KR, ER and DH domains suggest that these enzymes might be included in a group of highly reducing PKSs (Cox and Simpson, 2009). Similarly to NRPSSTE, this PKSSTE lacked the

thioesterase (TE) or Claisen Cyclase (CLC) domains responsible for release of the products. However, several highly reducing fungal PKS have been described lacking this kind of domains such as lovastatin nonaketide synthases of *A. terreus* which shows similar architecture to PKSSTE. This might preclude the implication in both cases of other endogenous TEs necessary for product release (Chiang et al. 2010).

Finally, a new ORF was identified located 435 bp downstream the *pksste* gene which predicted an amino acid oxidase. This gene did not seem to be related to OTA biosynthetic pathway.

The expression patterns of these three genes were consistent with a coordinated mode of regulation and correlated positively with OTA production. These expression patterns were analysed by real time RT-PCR in permissive conditions for OTA production. Specific protocols were developed for each gene (table 2). In all cases, relative quantification was carried out and the expression of the constitutive gene of ß-tubulin was used as normalizer. The efficiencies of amplification for the 4 set of primers used were between 99.6 and 100.4 % (table 2) and no primer-dimers were detected in any of the dissociation curves performed. Therefore, the three protocols described can be considered highly optimized. In all cases, the differences between the efficiency of *ß-tubulin* amplification and the others genes were less than 10%, therefore, the $2^{-\Delta\Delta CT}$ method was applied to analyse the expression of the three genes (Schmittgen and Livak, 2008). The study was performed using two *A. steynii* capable of producing OTA (Aso2 and 3.53) as well as a non-producing strain (CBS 112813) along 3-7 days of culture. The results indicated a coordinated pattern of expression for the three genes at high levels by the OTA-producing strains and the expression rate was positively related to OTA concentration detected in the media after 6 days of incubation (Figure 2). No expression was detected for any of the 3 genes in cultures of the OTA non-producing

401 strain at any time of incubation neither was OTA detected in the media. *A. steynii* Aso2
402 was the highest and more consistent OTA-producing strain (maximum OTA levels of
403 140.97 µg/g agar) and it also expressed the three genes at higher levels. This strain
404 achieved maximum expression levels of 361, 322 and 8374 times higher than the
405 control non-producing strain. *A. steynii* 3.53 strain also consistently expressed the three
406 genes (*p450ste*, *nrpsste* and *pksste*) 160, 104 and 3412 times, respectively, higher than
407 the expression of the non-producing strain and produced large amounts of OTA with
408 maximum levels of 100.81 µg/g agar. The expression of the three genes along the time
409 in each of the two OTA-producing isolate showed a positive and significant correlation
410 with Pearson's correlation coefficients between 0.97 and 0.99. This coordinated
411 regulation agreed with other clustered toxin biosynthetic genes described so far in other
412 species (Seo et al. 2001; Yu et al. 2004). As mentioned above, the high similarity of
413 *p450ste*, *nrpsste* and *pksste* genes with those reported in other OTA-producing
414 *Aspergillus* species from two different Sections, some of which were demonstrated to be
415 required for OTA biosynthesis *(pks* and *nrps* in *A. westerdijkiae* and *A. carbonarius*),
416 suggested that they might be homologous. Taking together all the evidences presented
417 here, it might be concluded that there is strong support for the involvement of *p450ste*,
418 *nrpsste* and *pksste* in OTA biosynthesis as well as for their clustered organization,
419 similarly to many other mycotoxin biosynthetic genes. This gene organization might
420 favour a more efficient regulation of the genes involved in these biosynthetic pathways
421 and the occasional transfer of the complete pathways (Miller and Linz, 2005) As we
422 have described, three similar ORFs were identified in An15c0250 contig, located the
423 right arm of chromosome 3 genomic sequence of *A. niger* CBS 513.88 strain (figure 1).
424 The relative order of the three genes and their reading frame in this contig was the same
425 as we have described for *A. steynii* in this work. Additionally, relative location of

426 *AcOTAnrps* and *ACpks* genes in *A. carbonarius* is also consistent with this picture
427 (Gallo et al. 2014). Therefore, this might preclude a general situation in *Aspergillus*
428 OTA-producing species at least in both Sections (Circumdati and Nigri). On other hand,
429 the analysis of the 3' flanking region of these genes differed in both species (*A. steynii*
430 and *A. niger*). In *A. niger* genome revealed a nitric oxide synthase ORF whereas a
431 putative amino acid oxidase gene sequence was observed for *A. steynii*. This suggested
432 that the genome location of the cluster might be different in both species. This situation
433 would not be new, since other mycotoxigenic biosynthetic clusters such as those
434 producing fumonisins have different location in closely related *Fusarium* species
435 (Proctor et al. 2009).
436 Relative position of the OTA biosynthetic genes described in *P. nordicum* (Geisen et al.
437 2006) does not agree with the situation described in the present work for *A. steynii* and
438 *A. niger*. Karolewiez and Geisen (2005) reported a correlative position of two genes
439 encoding a PKS (*otapksPN*) and a NRPS (*otanpsPN*) in the same region although these
440 authors did not described any P450 encoding gene. Moreover, the identity of the
441 genomic sequences of the genes identified by these authors and those described in this
442 work was extremely low (less than 15%). All these aspects might support the idea of a
443 convergent evolution of non-homologous gene clusters as suggested Geisen and
444 Schmidt-Heydt (2009).
445 The information reported in this work and the tools described will aid to develop more
446 efficient diagnostic methods, the identification of other potential OTA-producing
447 species improving prevention strategies to reduce OTA risk in agroproducts.
448 Additionally, they will contribute to a better understanding of the evolution and
449 regulation of mycotoxin production.
450

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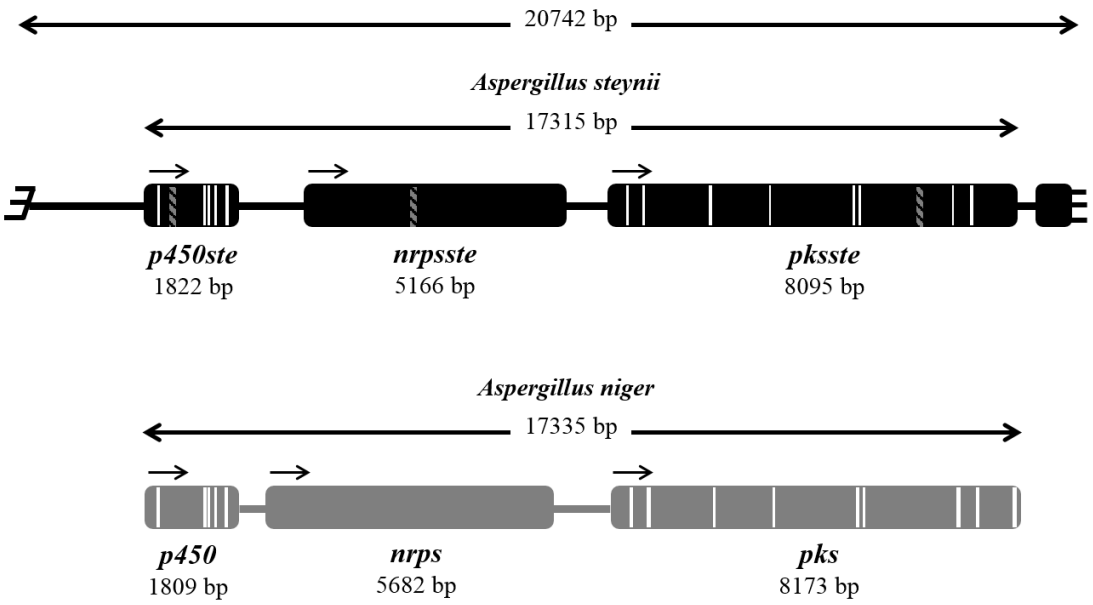
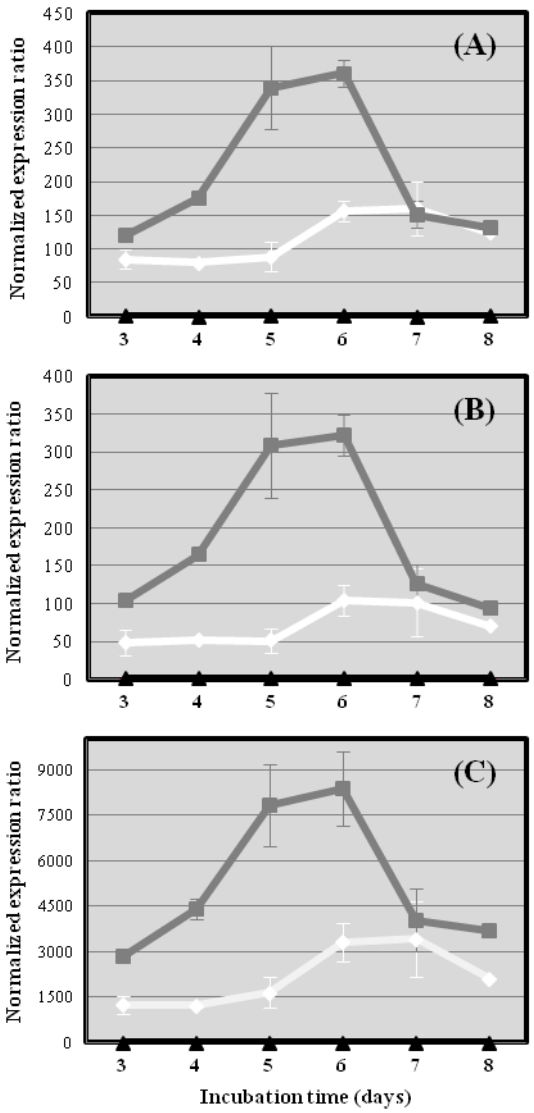
Figure legends

Figure 1. Scheme of the relative location of *p450ste*, *nrpsste* and *pksste* genes in the 20742 bp genomic region of *A. steynii* 3.53 (above). The same relative location of three putative genes encoding a P450, a NRPS and a PKS was also found in *A. niger* genomic contig An15c0250 (below). Black or grey rectangles represent the genes in *A. steynii* or *A. niger*, respectively. The white bars inside the genes correspond to the introns. The position of the introns was checked by sequencing cDNA in *A. steynii* whereas a bioinformatics approach was used to predict their position in *A. niger*. In *A. steynii* genomic region, the scratched bars inside the genes indicate the amplified region in real-time RT-PCR protocols. The black arrows indicate the direction of transcription of the genes. The size of each part is proportional to the length of the corresponding genomic region.

Figure 2. Time course relative expression of *p450ste* (A), *nrpsste* (B) and *pksste* (C) in CYA plates under permissive conditions measured by real time RT-PCR. In all cases the expression values of OTA producing strains (Aso2 and 3.53) were related to non-producing strain CBS 112813 after three days of incubation. Grey, white and black lines represent the results of Aso2, 3.53 and CBS 112813 respectively. Values are the average of two replicates with their corresponding standard errors.


20742 bp
Aspergillus steynii
17315 bp
p450ste
1822 bp
nrpsste
5166 bp
pksste
8095 bp
Aspergillus niger
17335 bp
p450
1809 bp
nrps
5682 bp
pks
8173 bp


(A)
Normalized expression ratio
450
400
350
300
250
200
150
100
50
0
3
4
5
6
7
8
(B)
Normalized expression ratio
400
350
300
250
200
150
100
50
0
3
4
5
6
7
8
(C)
Normalized expression ratio
9000
7500
6000
4500
3000
1500
0
3
4
5
6
7
8
Incubation time (days)

Direction	Round	Primer name	Primer sequence	Sequence Size
5'	1	GWGSP1-1 GWGSP2-1	5'-GAGACGCGTGCAAGCAGCCAGTTTA-3' 5'-TATGCGACAGCGGCGAAAAGAACAGCT-3'	1300 bp
	2	GWGSP1-2 GWGSP2-2	5'-GCTATCGCAATTGGACGGCAATGGAA-3' 5'-GCTCAGGTTGACTCCAACACCACCA-3'	1200 bp
3'	1	GWGSP1-1 GWGSP2-1	5'-CGACGAGTCCATCTTTCCGAAATCCCA-3' 5'-CATCCCGGACAGATGGACAGACCTTGA-3'	1600 bp
	2	GWGSP1-2 GWGSP2-2	5'-CTGATGCTCCTAGGAACTGCAACTTG-3' 5'-GGCGGTACAATGCTCGGACGGCTTGA-3'	800 bp
	3	GWGSP1-3 GWGSP2-3	5'-CCCTGTACATGAGCATCACCACCTA-3' 5'-CGCAGAAGCGAGCAACCTTTCAG-3'	1150 bp
	4	GWGSP1-4 GWGSP2-4	5'-GCGAGAATGCTAAAGCGCTGCTCT-3' 5'-GGCGATGAATACATCATGCAGAAACT-3'	1300 bp
	5	GWGSP1-5 GWGSP2-5	5'-CACGGAGAGTGCTTGCAAATTCGAA-3' 5'-TATTCTGATCGTTCATGCCCCAAGTT-3'	900 bp
	6	GWGSP1-6 GWGSP2-6	5'-GGCCAGTATGCCTCTGGAGCCAA-3' 5'-GCTATATGGCAGCTGCATCGATA-3'	1000 bp
	7	GWGSP1-7 GWGSP2-7	5'-CCTTCATGATACCAACAGCTTGGAT-3' 5'-CTGTCGAGGATACCACTTTCTGCA-3'	1100 bp
	8	GWGSP1-8 GWGSP2-8	5'- GGGAGACAGATGTTCCAAGCTTTGA-3' 5'-GGAGTCGAAATTTGAAATACTTGTGGT-3'	1500 bp
	9	GWGSP1-9 GWGSP2-9	5'-CTGGGTAGCCAAACCCGTGAGAC-3' 5'-CGCTTTATGTCGGGTCGGTGAAAT-3'	1500 bp
	10	GWGSP1-10 GWGSP2-10	5'-CAGCCCCAAGAACCTGACTCTGA-3' 5'-GGCAGACACGAGCACATTCTACGT-3'	1600 bp
	11	GWGSP1-11 GWGSP2-11	5'-GGAGATCGCTATCAAGGGTCTGGA-3' 5'-CCGACAGAGACCGACTCGTCAGA-3'	450 bp
	12	GWGSP1-12 GWGSP2-12	5'-CCTAGTCCGGGTTGGCTTGCAAAT-3' 5'-CCATTCTGGAGCAAAAGGTCGACTG-3'	700 bp
	13	GWGSP1-13 GWGSP2-13	5'-GGGTTTGGTTTGCTCCCAGGAT -3' 5'-GCGTAGCGTGGGAGCCCTATCG-3'	1250 bp
	14	GWGSP1-14 GWGSP2-14	5'-TCACCACGCGGCTGGAAGCAACG-3' 5'-CGCTGCCGTGCAAGTCGCTCGAT-3'	850 bp
	15	GWGSP1-15 GWGSP2-15	5'-CCTGGTTTGCGACGTGACCAACAA-3' 5'-GAAGCAGTGGCGCAGGCGATCAA-3'	1200 bp
	16	GWGSP1-16 GWGSP2-16	5'-CCATCAAAGCCAACTTTTCACAGCTT -3' 5'-GCTCCAGCATACTCAACCTCCTCA -3'	950 bp

Table 1. Primer sets designed to perform the genome walking approach to unravel the flanking regions of *p450ste* gene in *A. steynii* 3.53. The sequence of the primers used in each round is indicated as well as the size of the sequence obtained in each step.

Gene	Primer name	Primer sequence	Amplification efficiency (%)
ß-tubulin (*ßtub)*	BTUBQsteF BTUBQsteR	5'-GCCGTTCTCGTCGACCTTGAG -3' 5'-CTGACCGAAGACGAAGTTGTCG -3'	99.6 ± 0.80
Polyketide synthase (*pksste*)	PKSQsteF PKSQsteF	5'- TTGGCGATGCTGTGATGGCG -3' 5'-GCGACTGGAAGTGTAGCGGC -3'	100.0 ± 2.15
Cytochrome p450 monooxygenase (*p450ste*)	P450steF P450steR	5'-ACCATCGCTTACACAGACCTC-3' 5'-GGAGCGTGCAACCTCGTTCA-3'	99.8 ± 3.55
Non-ribosomal peptide synthetase (*nrpsste*)	NRPSsteF NRPSsteR	5'-CGTGCACGGCATTGCAAGAG -3' 5'-GGTCTCCCATGCTGCAAGGAA -3'	100.4 ± 0.40

Table 2. Primer set designed for real time RT-PCR quantification of the expression of the *A. steynii* genes described in this work. The name and sequence of the primers are indicated as well as amplification efficiencies calculated from the standard curve generated (indicated as the average of the two efficiencies calculated from DNAs from two strains ± standard error).